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ORIGINAL ARTICLE

Identification of differentially expressed genes in coronary atherosclerotic plaques from patients with stable or unstable angina by cDNA array analysis

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Summary. The composition of atherosclerotic plaques is a crucial factor in determining rupture, thrombosis and clinical events. In this study, we analyzed gene expression in coronary plaques from patients with stable or unstable angina using gene arrays. Total RNA was extracted from eight plaques collected by therapeutic directional coronary atherectomy. cDNA probes, generated by amplification, were hybridized to nylon arrays containing 482 genes. Here we report the results for the inflammation, adhesion and hemostasis subsets. Many genes not previously associated with atherosclerosis, such as the lymphocyte adhesion molecule MadCAM, were expressed in the plaques. ANOVA analysis showed higher tissue factor (TF) expression in unstable angina samples. Five genes were expressed at lower levels in unstable angina samples: anticoagulant protein S, cyclooxygenase (COX)-1, interleukin (IL)-7 and chemokines monocyte chemotactic protein (MCP)-1 and -2. Gene arrays provide a new approach to study plaque composition and identify candidate markers of plaque instability.

Keywords: angina, atherosclerosis, gene expression, molecular biology.

Introduction

Coronary heart disease is a complex and heterogeneous disease. Most acute coronary events result from rupture of the atherosclerotic plaque and super-imposed thrombosis [1]. Triggering of the acute coronary event depends on factors extrinsic to the plaque as well as on the susceptibility of the plaque itself (reviewed in [2]). The presence of inflammatory and prothrombotic mediators within the plaque plays a key role in plaque rupture and thrombosis [3]. Thus, defining the composition of a plaque is crucial to understand the mechanisms of plaque development and rupture. In order to identify new plaque components that may correlate with plaque instability, we have studied gene expression in coronary atherosclerotic plaques obtained by directional coronary atherectomy (DCA) from patients with stable or unstable angina, using sequence-verified cDNA arrays. We report here the results on 154 genes involved in inflammation and thrombosis.

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Materials and methods

Patient population

Twenty-seven patients with stable or unstable angina undergoing directional coronary atherectomy (DCA) were initially recruited for this study. Clinical and angiographic criteria were employed to classify the patients [4]. All patients gave written informed consent to participate in the study. All laboratory operators were unblinded only at completion of the study.

Coronary angiography and DCA

Selective coronary arteriography was performed in multiple views using the Judkin's technique. DCA was performed using standard clinical procedure.

Plaque handling and RNA extraction

The plaques extracted by DCA were flushed in RNAzol B^{TM} (Biogenesis, Poole, UK), immediately frozen in liquid nitrogen and stored until use. Plaques were disrupted on ice in 0.5 mL RNAzol B^{TM} and total RNA was extracted as per manufacturer's instructions. The RNA samples, re-suspended in the presence of 40 U RNase inhibitor (Boehringer Mannheim, Mannheim, Germany), were analyzed on a 1% agarose: MOPS gel stained with SYBR GOLD (Molecular Probes, Inc., Eugene,

OR). In four samples, no RNA was detectable. The remaining samples contained between <100 and 465 ng total RNA, however, in 15 samples partial RNA degradation was observed. Therefore only eight samples (four from stable angina and four from unstable angina patients) were accepted for further analysis. No correlation was observed between RNA amount or quality and plaque size or clinical group.

RNA amplification by SMART and preparation of probes

Due to the low amounts of total RNA available, probes were generated using cDNA amplification as described (SMART kit, Clontech, Pal Alto, CA, USA) [5]. ^{33}P-labeled probes were generated from 2 μL of amplified cDNA using Rediprime kit (Amersham Pharmacia, Bucks, UK).

Generation of the sequence verified cDNA array

From the IMAGE library [6], 1040 EST clones were selected, corresponding to 482 individual genes. Sequence-verified PCR products generated from the selected clones were gridded in quadruplicate onto nylon filters (Boehringer Mannhiem) using an automated 384-pin gridding device (Gentix, Hants, UK).

Hybridization, array scanning and image processing

Probes were hybridized to the cDNA arrays for 72 h at 45 °C. Following high stringency washes, the arrays were exposed to phospho-screens and images captured using a phosphoimager (Storm Scanner, Molecular Dynamics). The image analysis software DGENT (GlaxoWellcome) was used to detect the spot signal and to correct for local background.

Data processing and analysis

For each clone, the standard deviation (SD) of four replicated expression values was calculated. We determined the probability ($P < 0.01$) for each expression value to be above background or to be due to artefacts by performing a bootstrap analysis in S-plus, based on the values of control empty spots. To correct for possible differences in overall signal, the distributions of raw values across the arrays was normalized using the following formula:

$$\log(\text{observation}) - \left(\frac{\text{sum of logged observations for patient}}{\text{number of observations for patient}}\right)$$

ANOVA was performed blind across the two patient groups to identify genes that may be differentially expressed. To identify correlation within the gene matrix, cluster analysis was performed in SPSS using a between-group linkage-clustering algorithm on a Pearson correlation metric. The relationships between individual genes are displayed using a dendrogram with highly correlated genes grouped in nearby leafs [7].

Results

Genes common to plaques from patients with stable and unstable angina

The genes studied were selected because of their previous association with atherosclerosis or their possible relevance to the disease. The normalized data is represented graphically by means of a pseudo-color visualization tool (F. Falciani, unpublished data), based on the Eisen gene map [7]. The color intensity represents the magnitude of the expression: colors from blue to red indicate low to high expression levels, respectively, with 0 being the median intensity of the data set. Each row represents the normalized hybridization data from a single gene, and each column represents a patient (left, unstable angina; right, stable angina). The number of positive samples for each clone is reported in the last column.

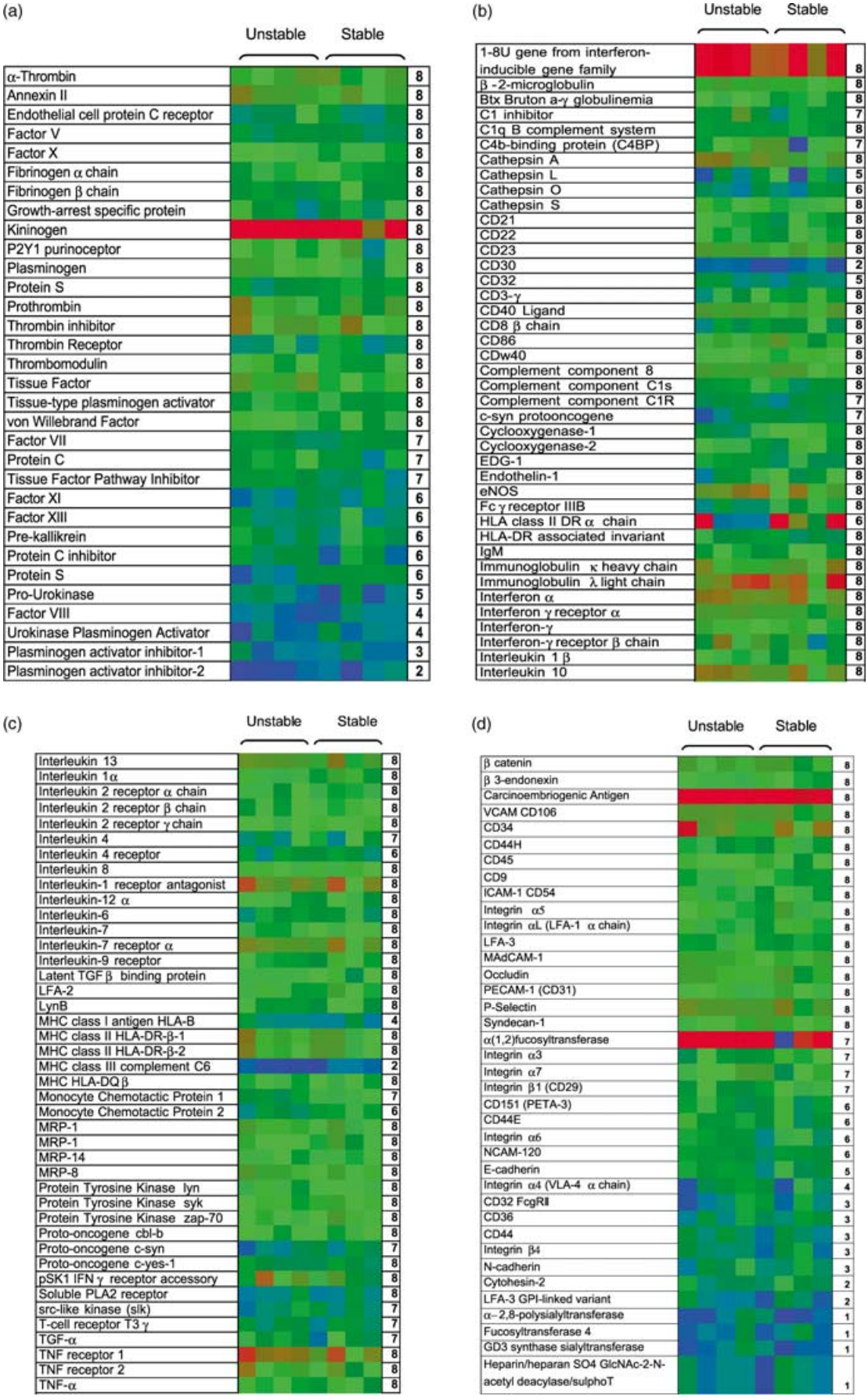
Haemostasis

The results for 32 genes encoding hemostasis components are shown in Fig. 1(a). Tissue factor (TF) was expressed in all samples as well as fibrinogen and thrombin. Both pro- and anticoagulant genes were identified. Interestingly, coagulation factors of hepatic synthesis, including factor VIII (FVIII), were expressed by cells within the plaques. Anticoagulant molecules such as protein C, protein S, thrombomodulin and tissue factor pathway inhibitor were expressed in most samples.

Inflammation and cell adhesion

The results for 84 genes encoding inflammation components are reported in Fig. 1 (b, c). Several pro-inflammatory molecules such as COX-2, IL-1α, IL-1β and TNF-α were found in the samples. All samples also expressed a number of molecules with anti-inflammatory activity: COX-1, IL-13, IL-10. Several genes, including CD25 and CD3, Syk and ZAP-70, indicated the presence of activated T lymphocytes. The presence of monocytes and endothelial cells in the samples was confirmed by the expression of well-known markers such as MCP-1 or CD31 (Fig. 1d). The expression of 38 genes involved in cell adhesion was studied. ICAM-1 and VCAM were found in all samples, but several molecules not previously linked to atherosclerosis were also identified (Fig. 1d). Particularly interesting

Fig. 1. Gene expression profile of human coronary plaque samples. The results for the genes involved in hemostasis (a), inflammation (b,c) and cell adhesion (d) are shown. A pseudo-color visualization matrix (see Methods) was used to provide a graphical representation of the primary normalized data. Colors from blue to red indicate low to high expression levels respectively; the color intensity represents the magnitude of the expression. In the matrix each row represents the normalized hybridization data from a single cDNA in the array, and each column represents a patient (unstable angina samples on the left, stable angina samples on the right). The number of positive samples for each gene is reported in the far right column.



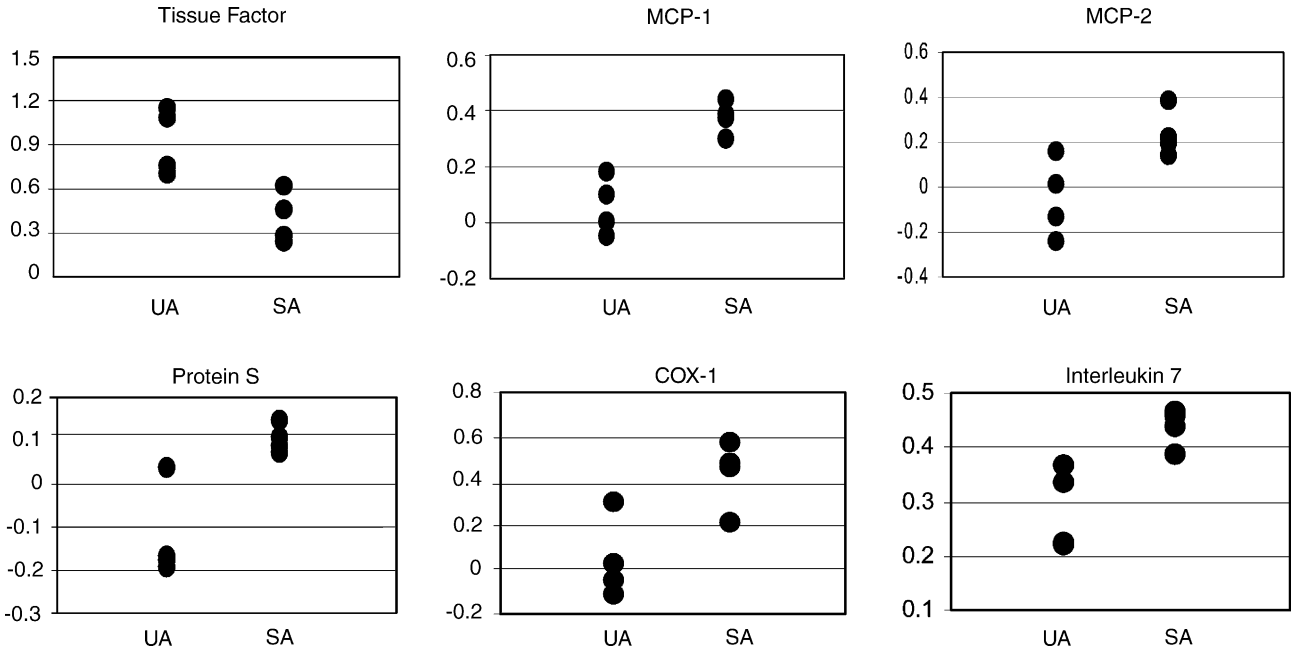


Fig. 2. Differentially expressed genes in stable and unstable angina plaque samples (ANOVA analysis). The results for TF, Protein S, COX-1, IL-7, MCP-1 and MCP-2 are shown. The data for each individual patient (unstable angina (UA) on the left, stable angina (SA) on the right) are shown in each graph.

was the expression in all samples of the mucosal addressin MadCAM, an endothelial adhesion molecule involved in lymphocyte homing [8].

Comparison of gene expression profiles in stable vs. unstable angina plaques

No significant differences were found in the total number of genes expressed in the two disease groups (data not shown). Within 109 genes representing hemostasis, inflammation and cell adhesion groups, six genes were expressed at significantly different levels between the two groups. The results are reported in Fig. 2. Tissue factor expression was significantly higher ($P = 0.0104$) in samples from unstable angina patients. Conversely, mRNA levels for five molecules were significantly higher in the stable angina patients: anticoagulant protein S ($P = 0.0069$); anti-inflammatory enzyme COX-1 ($P = 0.0174$); cytokine IL-7 ($P = 0.0111$); chemokines MCP-1 ($P = 0.0017$) and MCP-2 ($P = 0.03$).

Cluster analysis of hemostasis, cell adhesion and inflammation data

To identify possible relationships between the genes studied, a hierarchical clustering algorithm was used [7]. The dendrogram depicting the results of the cluster analysis is shown in Fig. 3. Distinct clones representing the same gene clustered in nearby leafs. Two main clusters and several subclusters were identified. The genes more highly expressed in stable angina samples were found clustered closely together (Fig. 3, upper insert). Several T-lymphocyte genes, such as *CD3, CD8*, c-*syn*, were found in the same cluster. Tissue factor, a marker of unstable angina, was found in the other main cluster (Fig. 3, lower insert), together with thrombomodulin, eNOS and annexin II, suggesting that the cluster could contain a prevalence of endothelial genes.

Discussion

To increase our knowledge on the composition of atherosclerotic plaques, and identify candidate markers of plaque instability, we studied the expression of 482 genes in human coronary atherosclerotic plaques using sequence-verified cDNA arrays. The major findings of this study, based upon genes involved in hemostasis and inflammation, are:

1. gene expression in coronary atherosclerotic plaques retrieved by DCA can be studied using gene arrays, by means of cDNA amplification
2. with this approach, genes previously not associated with coronary atherosclerosis have been identified
3. Differences in gene expression between plaques from patients with stable or unstable angina have been found.

Gene expressions analysis on DCA plaque samples

The study of gene expression in atherectomy samples presented some serious obstacles: despite careful handling, only less than a third of the plaque samples produced RNA of quality adequate for the study. However, the issue of low yield was overcome by using an amplification technique, an approach similar to the one recently reported by Zohlnhofer *et al*. on restenosis atherectomy samples [9].

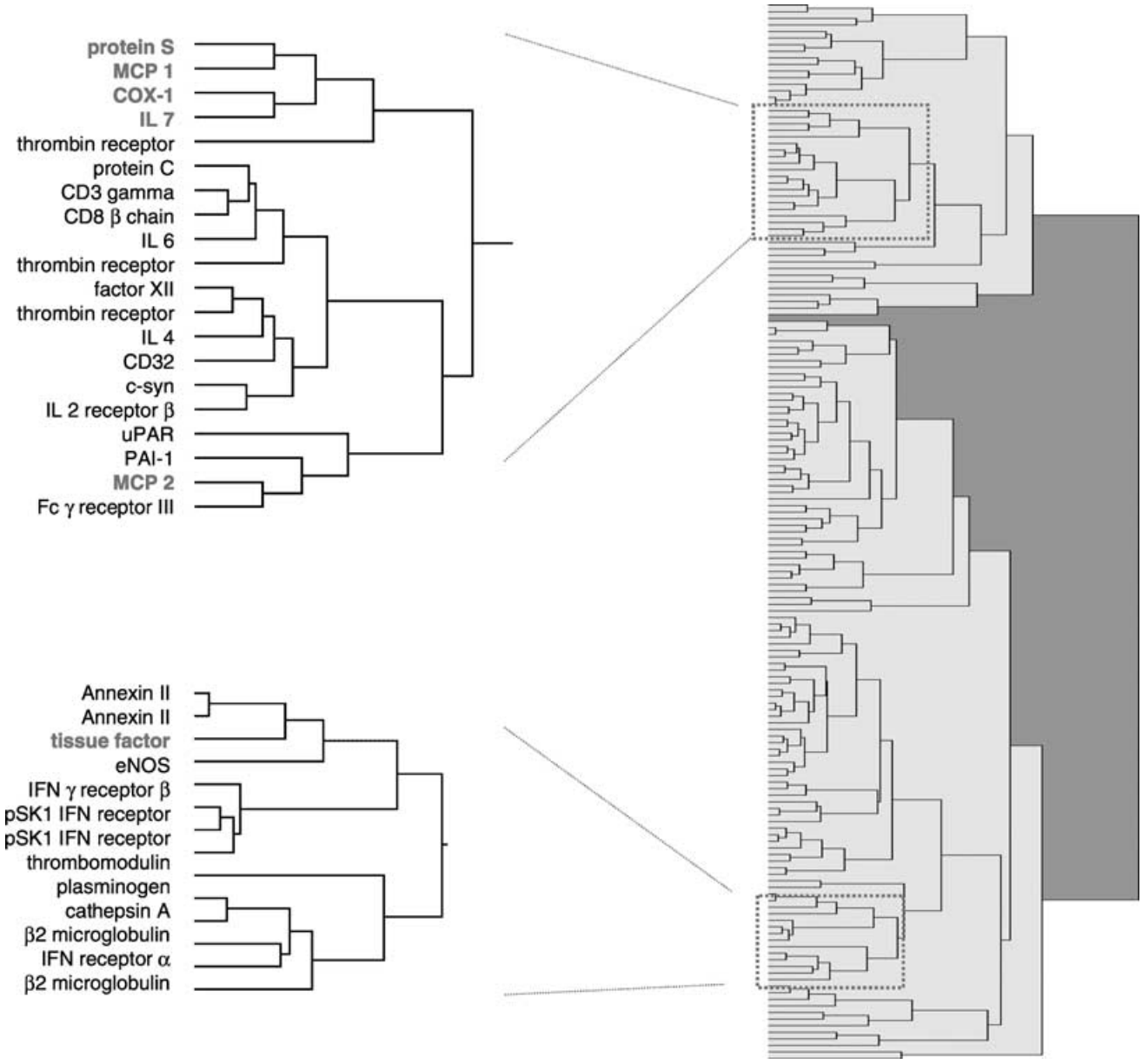


Fig. 3. Cluster analysis of combined data set. Cluster analysis was performed on the inflammation, cell adhesion and hemostasis data set. Genes in the same cluster are grouped on the basis of the pattern of expression across samples and have been shown to share common regulatory pathways, functional activities and/or cellular expression patterns. Two main clusters and several subclusters were identified. The genes that resulted significantly different in the two patient groups by ANOVA are in **bold**. The clusters containing the genes significantly higher in stable (top insert), or unstable (bottom insert) angina are shown. Duplicate clones of the same gene clustered in close proximity, supporting the correlation criteria. For many of the genes studied, more than one clone was present in the array, and the individual clones were kept separate for cluster analysis. Distinct clones representing the same gene clustered in nearby leafs, indicating that the effects of experimental noise or hybridization artefacts were limited.

Identification of new atherosclerosis-linked genes

The next major finding of this study is the identification of several genes not previously associated with atherosclerosis. Some of these genes were so far considered to be specific to other organs/tissues. Such findings are common to gene expression studies on gene arrays, and may be due to the sensitivity of the method [5]. Coagulation FVIII, found in four plaque samples, is normally expressed by hepatocytes and liver reticuloendothelial cells [10]. Elevated circulating levels of FVIII have been associated with increased risk of coronary heart disease [11] and diabetes [12]. FVIII antigen levels have been shown to correlate with a history of myocardial infarction and atheroma [13]. The finding of FVIII expression in plaque tissue raises the possibility that its local production may contribute to the thrombogenicity of the plaque.

Another intriguing finding of this study was the expression of MadCAM in all plaque samples. Several adhesion molecules have been detected in the atherosclerotic plaque and shown to play a key role in lesion progression [14]. MadCAM is

an endothelial adhesion molecule constitutively expressed in high endothelial venules in lymphatic organs such as tonsils and Peyer's patches in the intestine, and is involved in lymphocyte homing and leukocyte influx into mucosal inflamed tissue [8]. MadCAM expression has been detected in chronic inflammatory intestinal disorders [15–17]. This is the first report that links MadCAM expression to atherosclerosis; one of MadCAM counter-receptors, the integrin $\alpha 4\beta 1$, was also expressed in the plaque samples. MadCAM is specifically involved in T cell recruitment; interestingly, a large number of T cell genes were found in the plaque samples, suggesting that MadCAM may play a key role in T cell recruitment in the plaque.

Comparison of gene expression in stable vs. unstable angina

Very few studies compare gene expression in human coronary plaques from stable vs. unstable patients. TF is known to be expressed at higher levels in plaques from unstable angina patients [4], thus conferring increased thrombogenicity to the tissue; this finding was confirmed in our study (Fig. 2). Interestingly, mRNA levels for the anticoagulant protein S, cofactor for the protein C pathway, were lower in the unstable angina group. It is tempting to speculate that a local imbalance between pro-coagulant TF and anticoagulant molecules such as protein S could contribute to tilt the local hemostatic balance towards thrombosis. Four other genes were expressed at significantly higher levels in samples from stable angina patients: COX-1, MCP-1, MCP-2, IL-7. COX-1, expressed constitutively in endothelial cells and involved in maintaining vascular homeostasis [18], is likely to play a key role in protecting from plaque rupture, thrombosis and the consequent clinical events. This is supported by *in vivo* data, showing that increased expression of COX-1 in angioplasty-injured carotid arteries in a pig model was associated with inhibition of thrombosis [19]. The finding of higher COX-1 levels in stable plaque samples is the first data in man consistent with a protective role for COX-1 on plaque stability.

MCP-1 and MCP-2 are chemokines required for the recruitment of monocytes but also involved in immune responses and vascular remodeling [20]. This is the first report of MCP-2 expression in atherosclerosis, while MCP-1 has been extensively studied and shown to correlate with atherosclerosis development [21]. In our study, both chemokines were expressed at higher levels in stable angina samples. The significance of this finding is unclear, but suggests differences in the regulation of monocyte recruitment in stable and unstable plaques.

IL-7 is produced by a variety of cells and can act as a growth factor for mature T cells [22]. No data is available on the role of IL-7 in atherosclerosis development, however, its expression is consistent with the abundance of T cells observed in our samples. Recently IL-7 was shown to inhibit apoptosis of mature T cells, partly through the up-regulation of Bcl-2 family members [23]. IL-7 was expressed at higher levels in stable angina samples. Further studies will be required to investigate whether the presence of specific T cell subsets correlates with plaque stability. The correlation between IL-7 expression and several T cell markers (CD3, CD8, CD40, c-Syn) in the 'stable angina cluster' suggests that prolonged survival may be another mechanism of accumulation of T cells in plaques.

The limited number of patients in each group combined with the large number of genes studied does not allow for a very powerful statistical analysis; however, these results provide candidates for further investigation into the composition of plaques from stable and unstable angina. No confirmation of the RNA results was performed using a secondary method because of the limited amount of sample material. However since the completion of this study, the same cDNA arrays have been used to investigate gene expression in *in vitro* cellular models, and the results obtained on the gene arrays have been confirmed at the mRNA as well as protein level, thus supporting the validity of this approach [24]. This study is to our knowledge the first large-scale approach to studying composition of coronary plaques. These results provide a unique prospective on coronary atherosclerosis and suggest new hypothesis on local mechanisms that may be involved in plaque rupture and thrombosis.

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